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(54) Title: METHOD OF PROTEIN AND PEPTIDE CLEAVAGE

(57) Abstract

In a method of cleaving a protein or peptide in an Asn-Gly bond thereof, the protein or peptide is treated with a compound of the general formula (I): R_1-$(CH_2)_n$-NH-$(CH_2)_m$-R_2, wherein R_1 is NH_2 or OH, R_2 is hydrogen, lower alkyl, NH_2, OH or halo, n is an integer from 1 to 3, and m is 0 or an integer from 1 to 3.

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METHOD OF PROTEIN AND PEPTIDE CLEAVAGE

The present invention relates to the cleavage of proteins and peptides, especially the cleavage of fusion proteins and peptides obtained in recombinant DNA methods.

When producing peptides and proteins in bacteria by recombinant DNA techniques, it is often necessary to produce the peptide or protein fused to a bacterial protein in order to protect the protein or peptide against degradation by proteases, and eventually cleave the fusion product to recover the desired protein or polypeptide. The selection of specific reagents for such cleavage has so far been very limited. One attractive way of cleavage has been the use of hydroxylamine, which cleaves between the amino acids asparagine (Asn) and glycine (Gly).

A drawback with hydroxylamine is, however, that in addition to cleaving the Asn-Gly bond, it also reacts with other asparagine and glutamine residues in the peptide or protein, resulting in the formation of hydroxamates (Canova-Davis, E., et al., (1992) J. Biochem. 285, 207-213). These hydroxamates are difficult to separate from the "native" peptide or protein, and it is also difficult to analyse the content of hydroxamates in a peptide or protein product, since the formation of a hydroxamate of the peptide or protein only represents a mass change of 16 daltons an no change in charge.

The object of the present invention is to provide a cleaving agent which is devoid of the above disadvantages. In accordance with the present inventive concept, such a cleaving agent would, on one hand, like hydroxylamine, act as a nucleophile in the desired cleavage reaction, but would, on the other hand, (i) be heavier than hydroxylamine such that, in the case of side reactions similar to the hydroxamate formation, these side reaction products would be easier to detect, and (ii) carry a charge such that the reaction products would be easier to separate.

According to the present invention, it has been found that specific cleavage of Asn-Gly bonds in proteins and

Germany), 1 mM EDTA, 10 % ethanol, 3 M guanidine-HCl, pH 9.4, at 45 °C for 6 to 18 h.

Analytical HPLC was then performed in the same way as for the hydroxylamine cleavage mixture above and resulted in the same fast eluting peak (Apo fragment 185-243) being obtained.

EXAMPLE 2

Cleavage of Insulin-like Growth Factor I

Insulin-like Growth Factor 1, hereinafter IGF-I, was produced in E. coli as a fusion protein Z-IGF-I, where Z is a modified domain of Staphylococcus protein A, essentially as described in Moks, T., et al., (1987) Bio/Technology 5, 379-382. The cleavage site between Z and IGF-I is Asn-Gly, which site was cleaved with (A) hydroxylamine and (B) ethylenediamine as described below.

A. hydroxylamine cleavage (comparative)

Cleavage with hydroxylamine of 100 µg of Z-IGF-I was performed under the conditions specified for the corresponding cleavage of Apo in Example 1 above.

After cleavage, IGF-I was purified by desalting to 0.2 M acetic acid on PD10 columns (Pharmacia LKB Biotechnology AB, Sweden).

Desalting was followed by cation exchange on a MonoS column no. 2623 (Pharmacia LKB Biotechnology AB, Sweden). The gradient used was 10-52 % B in A for 20 minutes, where A is: 20 mM ammonium acetate, 10% ethanol, pH 5.4, and B is: 1 M ammonium acetate, 10 % ethanol, pH 5.4.

Analytical HPLC was then performed on a Hewlett Packard 1090 using a YMC-Pack Protein-RP column, PRRP-03-5, C4, 5 µm, 250 x 4.6 mm (YMC Inc., USA) and a gradient of 30-40 % B in A over 40 min. at ambient temperature, where A is: 0.25 % PFPA in MilliQ water, and B is: 0.25 % PFPA in acetonitrile (HPLC quality). The flow was 1.0 ml/min..

B. Ethylenediamine cleavage (method of invention)

100 µg of Z-IGF-I were cleaved with ethylenediamine under the same conditions as those specified for the ethylenediamine cleavage of Apo in Example 1.

Samples were taken after 2, 4 and 6 h and directly analysed, after desalting and cation exchange as described above for the hydroxylamine cleavage of Z-IGF-I, by HPLC on a Beckman System Gold HPLC (Beckman, USA) and a Brownlee Aquapore Butyl column, 7 µm, 220 x 4,6 mm (Applied Biosystems, USA) with a gradient 26-31 % B in A over 30 min. at 50 °C, where A is: 0.25 % PFPA in MilliQ water, and B is: 0.25 % PFPA in acetonitrile (HPLC quality). The flow was 1.0 ml/min..

IGF-I was detected after 2 h, and at 4 h the IGF-I peak had increased by about two times. The IGF-I peak at 6 h was about the same size as that for the sample taken at 4 h.

The yield of ethylenediamine cleavage after 4 h was 25 % compared with that obtained in the optimized hydroxylamine cleavage.

Pure IGF-I was added to the 4 h sample, which was then analysed by HPLC. The IGF-I peak increased as assumed.

IGF-I from the ethylenediamine cleavage was isolated by semipreparative HPLC in the same way as for the analytical HPLC described above. The IGF-I fraction produced was then N-terminal sequenced in five steps by Edman degradation on a solid phase sequencer, Prosequenator 6600 (Millipore, USA). Carboxyl groups on the peptides were covalently bound to arylamine membrane (Millipore) prior to sequencing. The sequence obtained was identical with that of IGF-I, NH2-GlyProGluThrLeu and no additional sequences could be detected.

The molecular weight of the peptides were determined on a plasma desorption time of flight mass spectrometer, PDMS (Perkin Elmer, USA, BioIon, Sweden) or on an ESR spectrometer (VG Quattro, Fisons, England). The resulting mass spectra gave a single peak at Mw 7646 (theor. Mw 7649). No peak at Mw 7690 was detected which should have been the case if ethylenediamine had reamidated with asparagine or glutamine.

EXAMPLE 3

Cleavage of Z-IGF-I with 2-ethylaminoethylamine

100 µg of the fusion protein Z-IGF-I were cleaved with 2-ethylaminoethylamine (Mw 88.15) under the same conditions as for the ethylenediamine cleavage of Z-IGF-I described in Example 2, except that the reaction temperature was decreased to 34 °C, CAPSO buffer, pH 9.3, (Sigma, USA) was used instead of Tris, and the reaction time was prolonged to 18 h.

Analysis by HPLC on a Hewlett Packard 1090, using a YMC-Pack Protein-RP column, PRRP-03-5, C4, 5 µm, 250 x 4.6 mm (YMC Inc., USA) and a gradient 30-40 % B in A, where A is: 0.25 % PFPA in MilliQ water, and B is: 0.25 % PFPA in acetonitrile (HPLC quality), over 30 min. at ambient temperature and a flow of 1.0 ml/min. indicated a yield of 45 % compared to hydroxylamine cleavage.

At these conditions, the yield of IGF-I, cleaved with ethylenediamine, was 32 % compared to hydroxylamine.

IGF-I from the cleavage reaction was purified via gel filtration, anion exchange and reversed phase HPLC. The mass spectra was identical to that of IGF-I and no peak at 7690 corresponding to reamidation could be detected.

EXAMPLE 4

Cleavage of Z-IGF-I with 2-(2-aminoethylamino)ethanol

Cleavage of Z-IGF-I with 2-(2-aminoethylamino)ethanol at the same conditions as in Example 3 gave a yield of 28 % compared to hydroxylamine cleavage. Mass spectra after purification of IGF-I as in Example 3 was identical to that of IGF-I and no reamidation products could be detected.

CLAIMS

1. A method of cleaving a protein or peptide in an Asn-Gly bond thereof, **characterized** by treating the protein or peptide with a compound of the general Formula I:

$$R_1 - (CH_2)_n - NH - (CH_2)_m - R_2 \qquad (I)$$

wherein

R_1 is NH_2 or OH,
R_2 is hydrogen, lower alkyl, NH_2, OH or halo,
n is an integer from 1 to 3, and
m is 0 or an integer from 1 to 3.

2. The method of claim 1, **characterized** in that R_1 is NH_2 and R_2 is hydrogen, methyl, NH_2, OH, fluoro, chloro or bromo.

3. The method of claim 2, **characterized** in that the compound of formula I is selected from ethylenediamine, 2-ethylaminoethylamine and 2-(2-aminoethylamino)ethanol.

4. The method of any one of claims 1 to 3, **characterized** in that said compound of Formula I is dissolved in a reaction medium.

5. The method of any one of claims 1 to 3, **characterized** in that said compound of Formula I is immobilized to a solid phase.

6. The method of any one of claims 1 to 5, **characterized** in that the cleavage reaction is carried out in an aqueous or aqueous ethanolic medium.

7. The method of any one of claims 1 to 6, **characterized** in that said protein is a fusion protein having an Asn-Gly bond in the link between the fusion partners.

8. Use of a compound of the general Formula I:

$$R_1 - (CH_2)_n - NH - (CH_2)_m - R_2 \qquad (I)$$

wherein

R_1 is NH_2 or OH,

R_2 is hydrogen, lower alkyl, NH_2, OH or halo,

n is an integer from 1 to 3, and

m is 0 or an integer from 1 to 3,

for cleaving proteins and peptides.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/00349

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 1/107, C12N 15/62
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, WPIL, US PATENTS FULLTEXT DATABASES, MEDLINE, BIOSIS, EMBASE, SCISEARCH, DIALINDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	INT.J.BIOCHEM., Volume 15, No 7, 1983, Kia-Ki Han et al, "Current developments in chemical cleavage of proteins" page 875 - page 884 --	1-8
A	ANALYTICAL BIOCHEMISTRY, Volym 154, 1986, Said Mahboub et al, "Applications of Chemical Cleavage Procedures to the Peptide Mapping of Neurofilament Triplet Protein Bands in Sodium Dodecyl Sulfate-Polyacrylamid Gel Electorphoresis", page 171 - page 182 --	1-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, file 155, Medline, Dialog accession no. 00851695, Medline accession no. 68411695, SAKIYAMA F: "Chemical cleavage of peptide bonda", Tanpakushitsu Kakusen Koso (Japan) Jan 1968, 13 (1) p 1-10 --	1-8
A	Dialog Information Services, file 157, Aidsline, Dialog accession no. 00003217, Shieh TC et al: "Purification of Recombinant HIV-TAT Protein from E Coli (Meeting Abstract)", Faser J; (7):A2252 1990 ISSN 0892-6638 --	1-8
A	Dialog Information Services, file 73, Embase, Dialog accession no. 104771, Embase accession no. 74106547, Oh S.K. et al: "Peptide bond hydrolysis by hydroxoaquo ethylenediamine diacetatocobalt(III) --	1-8